FUNDAMENTAL AND APPLIED TOXICOLOGY 15, 536–557 (1990)

Risk Extrapolation for Chlorinated Methanes as Promoters vs Initiators of Multistage Carcinogenesis

KENNETH T. BOGEN[1]

Lawrence Livermore National Laboratory, University of California, Livermore, California 94550

Received September 25, 1989; accepted May 11, 1990

Risk Extrapolation for Chlorinated Methanes as Promoters vs Initiators of Multistage Carcinogenesis. BOGEN, K. T. (1990). *Fundam. Appl. Toxicol.* 15, 536–557. "Cell-kinetic multistage" (CKM) models account for clonal growth of intermediate, premalignant cell populations and thus distinguish somatic mutations and cell proliferation as separate processes that may influence observed rates of tumor formation. This paper illustrates the application of two versions of a two-stage CKM model (one assuming exponential and the other geometric proliferation of intermediate cells) for extrapolating cancer risk potentially associated with exposure to carbon tetrachloride, chloroform, and dichloromethane, three suspect human carcinogens commonly present in trace amounts in groundwater supplies used for domestic consumption. For each compound, the models were used to calculate a daily oral "virtually safe dose" (VSD) to humans associated with a cancer risk of 10^{-5}, extrapolated from bioassay data on increased hepatocellular tumor incidence in B6C3F1 mice. Exposure-induced bioassay tumor responses were assumed first to be due solely to "promotion" (enhanced proliferation of premalignant cells, here associated with cytotoxicity), in accordance with the majority of available data on *in vivo* genotoxicity for these compounds. Available data were used to model dose response for induced hepatocellular proliferation in mice for each compound. Physiologically based pharmacokinetic models were used to predict the hepatotoxic effective (metabolized) dose as a function of parent compound administered dose in mice and in humans. Resulting calculated VSDs are shown to be from three to five orders of magnitude greater than corresponding values obtained assuming each of the compounds is carcinogenic only through induced somatic mutations within the CKM framework. Key issues and uncertainties in applying CKM models to risk assessment for cancer promoters are discussed.

Multistage cancer risk models that account for clonal growth of intermediate (initiated, premalignant) cell populations, or "cell-kinetic multistage" (CKM) models, have long been successfully used to describe age-specific incidence trends for human cancer and experimentally induced tumors in animals (Armitage and Doll, 1957, 1961). More recently, a two-stage stochastic model allowing for birth and death of initial and intermediate cells has been used to model human cancer incidence and has also been proposed to describe spontaneous and induced carcinogenesis in general (Moolgavkar and Venzon, 1979; Moolgavkar *et al.*, 1980; Moolgavkar and Knudson, 1981). There is growing interest in applying such a model to the problem of predicting human cancer risk posed by exposure to environmental agents (Moolgavkar, 1983; Thorslund *et al.*, 1987; Swenberg *et al.*, 1987; Moolgavkar *et al.*, 1988; Wilson, 1988; Dewanji *et al.*, 1989; Bogen,

[1] To whom all correspondence should be addressed at Environmental Sciences Division (L-453), Lawrence Livermore National Laboratory, University of California, P.O. Box 808, Livermore, CA 94550.

0272-0590/90 $3.00

1989). Because these models distinguish between mutations and cell population kinetics as separate processes that influence the rate of tumor formation, such models can predict widely different cancer risks for agents affecting only one process (e.g., "promotion" via induced net cell proliferation assuming a threshold- or quasithreshold-type dose response) but not the other (e.g., somatic mutatioin assuming a no-threshold dose response). Such an approach allows for distinct treatment of genotoxic carcinogens and nongenotoxic tumor promoters, provided that generally acceptable empirical criteria for the latter classification can be specified. This approach has been advocated (Williams, 1967; Weisburger and Williams, 1983; Wilson, 1988) but is currently not specifically incorporated into widely used procedures for cancer risk assessment (U.S. EPA, 1986).

Specific proposals for incorporating information on induced cell proliferation into quantitative risk assessment for cancer promoters currently focus on the use of traditionally defined no-observed-effect levels and safety factors (Weisburger, 1988; Clayson *et al.*, 1989; Butterworth, 1989). In contrast, this paper illustrates a more rigorous approach whereby an approximate two-stage CKM model[2] (Armitage and Doll, 1957; Moolgavkar, 1983; Thorslund *et al.*, 1987) is incorporated along with experimental data on tumor induction and induced cellular proliferation into a risk extrapolation procedure for promoters of hepatocellular carcinoma in mice. Because this approximate CKM model assumes that premalignant cell clones grow exponentially *in vivo*, a new version of this model (Bogen, 1989), using the biologically more plausible assumption of geometric cell proliferation, was also applied in the present analysis. The risk extrapolation procedure was applied to the chlorinated methanes, carbon tetrachloride (CCl_4), chloroform ($CHCl_3$), and dichloromethane (methylene chloride, CH_2Cl_2), three compounds known to be hepatocarcinogenic to mice and also known (as discussed below) to be inefficient or ineffective at inducing genotoxicity in mammalian cells *in vivo*.

In the following analysis, all three compounds are first assumed to be "pure promoters" within the CKM framework, an assumption that is suggested (but by no means proven) by data indicating minimal or nonexistent genotoxicity of these compounds in mammalian cells *in vivo* coupled with an ability to induce hepatocellular toxicity and proliferation (discussed below). Thus, in the first part of this analysis, the compounds are presumed ineffective at producing mutations assumed to be necessary in a CKM carcinogenic process; rather, they are assumed to be effective, at cytotoxic doses, in selectively increasing the rate of net cellular proliferation within premalignant cell clones. Available data are used to model the dose–response relationship for compound-induced cell proliferation in mice, and this relationship is assumed to be the same for humans. Increased predicted cell proliferation is then presumed to be proportional to increased growth parameter values in the CKM models fit to corresponding sets of mouse bioassay data on liver tumors induced by these compounds. For each compound, CKM models are then used to extrapolate a corresponding "virtually safe dose" (VSD) associated with a predicted increased cancer risk to humans of 10^{-6}.

[2] The approximate CKM model referred to here is based on a "semistochastic" two-stage CKM model in which interstage transition via somatic mutation is modeled as a stochastic process, but in which proliferation of premalignant cell clones is modeled as deterministic exponential growth. In the "fully stochastic" two-stage CKM model proposed by Moolgavkar *et al.* (see references cited), both the transition and proliferation processes are modeled as stochastic birth-and-death processes. Approximate forms of the semistochastic model are considered here to illustrate the application of CKM models to quantitative risk assessment for cancer promoters. However, these approximations are identical to analogous approximations for the corresponding "fully stochastic" model (see Appendix).

In the second part of this analysis, the VSDs calculated using CKM "promotion" models are compared to values calculated using corresponding CKM models under the assumption that the compounds considered are purely mutagens (and not promoters) in the CKM context. In applying both the CKM promotion-only and mutation-only models, appropriate physiologically based pharmacokinetic (PBPK) models are used throughout to predict the cytotoxic or genotoxic effective (metabolized) dose to liver in mice and in humans as a function of applied dose of parent compound. Finally, the calculated CKM-based VSDs are compared to VSDs based on the same mouse bioassay data sets but calculated using the "linearized" multistage model currently used in many regulatory cancer risk assessments, and issues concerning the application of the outlined procedure to cancer risk assessment are discussed.

Hepatocarcinogenicity of Chlorinated Methanes in B6C3F1 Mice

Bioassay data. Experimentally induced carcinogenic effects associated with lifetime exposure to CCl_4, $CHCl_3$, and CH_2Cl_2 in rodents have implicated these compounds as probable or potential human carcinogens (IARC, 1979a,b, 1982, 1986; U.S. EPA, 1984, 1985a,b,c, 1987). The present CKM-based cancer risk extrapolation was based on bioassay data on hepatocarcinomas (plus adenomas for CH_2Cl_2) in control and low-dose female B6C3F1 mice in studies by the National Cancer Institute (NCI, 1976a,b, 1977; Reuber, 1979) and the National Toxicology Program (NTP, 1986), as summarized in Table 1. With regard to the bioassay data for CCl_4, note that because 100% incidence was observed in the 40 low-dose female mice used and thus lower dose rates might also have yielded a 100% incidence, these data cannot be used to derive a point estimate of hepatocarcinogenic potency for CCl_4 in mice. Nevertheless, the data suggest that an oral dose of 1250 mg/kg CCl_4 on the NCI dosing regimen would yield a cumulative liver cancer risk greater than (40–1)/40 or 97.5% by 97 weeks of age in these mice. Under this assumption, lower-bound CKM-based risk extrapolations were undertaken for CCl_4 based on the NCI mouse bioassay data.

Information Concerning Likely Mechanism of Carcinogenic Action

CCl_4. Most assays of CCl_4's mutagenicity have been negative, particularly those involving mammalian systems (U.S. EPA, 1984). *In vivo* assays of hepatic DNA damage using rats exposed to CCl_4 have been uniformly negative (Mirsalis and Butterworth, 1980; Mirsalis *et al.*, 1982; Bermudez *et al.*, 1982). In contrast, CCl_4 is a classic agent for inducing hepatic necrosis and regenerative hepatocellular proliferation (Leevy *et al.*, 1959; Dinman *et al.*, 1963; Schultze *et al.*, 1973) and has been shown to induce in exposed mice a substantial increase in the percentage of hepatocytes in S-phase compared to that in unexposed mice (Mirsalis *et al.*, 1985; Doolittle *et al.*, 1987). Chemically induced hepatocarcinogenesis has been shown to be promoted by necrogenic doses of CCl_4 in B6C3F1 mice and in hamsters (Dragani *et al.*, 1986; Tanaka *et al.*, 1987); in mice this has been shown to be associated with increased general hepatocellular proliferation plus decreased cytotoxicity specifically to neoplastic hepatocellular nodules, i.e., with increased net proliferation of premalignant hepatocyte clones (Dragani *et al.*, 1986). A number of effective tumor-promoting compounds, including phorbol esters and CCl_4, have also been shown to be potent activators of intracellular protein kinase C, an enzyme thought to be involved in regulating cell growth (Roghani *et al.*, 1987).

$CHCl_3$. Most genotoxicity assays for $CHCl_3$ have yielded either negative or incon-

INPUT

Compound

CCl_4

$CHCl_3$

CH_2Cl_2

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[b] CCl_4, dose (
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TABLE 1

INPUT PARAMETER VALUES FOR CKM MODELS OF HEPATOCELLULAR CARCINOMA[a] INCIDENCE IN MICE EXPOSED TO CHLORINATED METHANES

Compound	Applied dose[b]	Estimated effective dose, d^c (mg/kg liver)	Input parameter values[d]					
			t_1 (year)	t_2 (year)	t_3 (year)	R_0 (%)	R_d (%)	K_φ (year^{-2} $\times 10^{-6}$)
CCl_4	1250	21.1	$\frac{7}{52}$	$\frac{85}{52}$	$\frac{97}{52}$	1.25	>97.5	1500
$CHCl_3$	230	2230.	$\frac{5}{52}$	$\frac{83}{52}$	$\frac{97}{52}$	1.25[e]	88.9	1500
CH_2Cl_2	2000	1210	$\frac{8.5}{52}$	$\frac{110.5}{52}$	$\frac{112.5}{52}$	6.0	34.8	1750

[a] Hepatocellular carcinoma or adenoma in the case of CH_2Cl_2.

[b] CCl_4, dose (in mg/kg) to 28-g female B6C3F1 mice was administered orally 5 days/week for 78 weeks in corn oil vehicle (NCI, 1976a, 1976b, 1977). $CHCl_3$, dose (in mg/kg) to 30-g female B6C3F1 mice was administered orally 5 days/week for 78 weeks in corn oil vehicle (NCI, 1976a; Reuber, 1979). CH_2Cl_2, dose (in ppm) to 32-g female B6C3F1 mice was administered in respired air 6 hr/day, 5 days/week for 102 weeks (NTP, 1986).

[c] Toxicology effective dose to hepatocytes (total metabolized dose 1 hr postexposure for CCl_4 and $CHCl_3$, total daily GST-metabolized dose to liver for CH_2Cl_2) was estimated using physiologically based pharmacokinetic models described in the text.

[d] See text for explanation of CKM dose–response models and their input parameters.

[e] Control hepatocellular carcinoma incidence was 0/19; R_0 was assumed to be equal to the value pertaining to CCl_4.

clusive results (Bridges *et al.*, 1981; Brookes and Preston, 1981; U.S. EPA 1985a; Rosenthal, 1987). In contrast, hepatotoxicity has been observed to occur in male and female B6C3F1 mice chronically exposed to oral doses of $CHCl_3$ in corn oil or in water (Bull *et al.*, 1986). Hepatocellular regeneration was found to be induced by this compound in liver and kidney of male B6C3F1 mice exposed to single oral doses, as measured by relative DNA incorporation of [^{3}H]thymidine (Reitz *et al.*, 1982). A similar finding was observed for male Swiss albino mice exposed to single oral doses of chloroform in corn oil and toothpaste vehicles (Moore *et al.*, 1982). Pereira *et al.* (1984) also found replicative hepatocellular DNA synthesis, measured by [^{3}H]thymidine incorporation into DNA, to be enhanced in male B6C3F1 mice after exposure to single oral $CHCl_3$ doses. Like CCl_4, $CHCl_3$ has been shown to be a potent activator of protein kinase C (Roghani *et al.*, 1987).

CH_2Cl_2. With the exception of a few reports of induced chromosome damage *in vitro* (Jongen *et al.*, 1981; Thilagar and Kumaroo, 1983; Thilagar *et al.*, 1984), this compound has generally not been found to be genotoxic to mammalian cells *in vitro* (Gocke *et al.*, 1981; Perocco and Prodi, 1981; Jongen *et al.*, 1981; Andrae and Wolff, 1983; Burek *et al.*, 1984). It also has been repeatedly shown to be negative in *in vivo* assays of DNA damage (Burek *et al.*, 1984; Sheldon *et al.*, 1987; Trueman and Ashby, 1987; Green *et al.*, 1988; Raje *et al.*, 1988; Ottenwalder and Peter, 1989), except for one recent report of slightly increased DNA damage measured by alkaline elution of DNA from hepatocytes of rats orally exposed to 2.55 g/kg CH_2Cl_2 in corn oil over 24 hr (Kitchin and Brown, 1989). In contrast, chronic exposure of rats or mice to this compound at levels as low as 100 to 500 ppm by inhalation or 170 (rats) to 590 (mice) mg/kg/day by ingestion in drinking water is known to cause mild hepa-

tocellular and renal toxicity (Klassen and Plaa, 1966; Weinstein *et al.*, 1972; U.S. EPA, 1985b; Kirschman *et al.*, 1986; Nitschke *et al.*, 1988), although CH_2Cl_2 is known to be much less hepatotoxic than CCl_4, $CHCl_3$, and several chlorinated ethanes (Klassen and Plaa, 1966). Although chronic exposure of female Sprague–Dawley rats to concentrations up to 500 ppm in air resulted in no evidence of increased hepatocellular DNA synthesis (Nitschke *et al.*, 1988), single inhalation exposures of B6C3F1 mice to 4000 ppm CH_2Cl_2 did result in a small but statistically significant increase in the percentage of hepatocytes in S-phase in these animals (LeFevre and Ashby, 1989). Like CCl_4 and $CHCl_3$, CH_2Cl_2 has been shown to be a potent activator of protein kinase C (Roghani *et al.*, 1987).

METHODS

PBPK Models

CCl_4. Metabolism of this compound is known to be the cause of its hepatotoxicity in rats (Reynolds, 1967; Bruckner *et al.*, 1986; Shertzer *et al.*, 1987, 1988; Labbe *et al.*, 1987) and in one study the rate of production of CO_2 (the primarily metabolite in rats) within 1 hr of dosing was shown to be the measure of CCl_4 metabolism most highly correlated ($R^2 = 0.88$) with the observed degree of enzyme-assayed liver injury (Reynolds *et al.*, 1984). Therefore, in the present analysis, the total amount of CCl_4 metabolized in 1 hr postexposure was assumed to be an appropriate measure of hepatotoxic effective dose. To estimate uptake and metabolism of CCl_4 in mice and humans, the PBPK model of Paustenbach *et al.* (1988), based on the four-compartment model developed by Ramsey and Andersen (1984), was adapted with the following modifications. CCl_4 metabolism in rats was assumed to occur by a single saturable pathway with V_{max} equal to 0.14 mg/hr for a 225-g rat (Gargas *et al.*, 1986a). (Corresponding V_{max} values scaled, using the methodology of Paustenbach *et al.* (1988), for modeling 30-g mice and 70-kg humans were 0.034 and 7.78 mg/hr, respectively.) Blood/air and tissue/air partition coefficients for rats and humans used were those of Paustenbach *et al.* (1988); those for mice were set equal to those for rats. All other physiological parameter values were taken to be the reference values reported by the U.S. EPA (1988), except that the latter reported alveolar ventilation rate for 70-kg humans was increased from 5.0 to a more realistic 5.6 liters/min. PBPK parameter values for mice and humans with weights different from those above were scaled by the method described by Paustenbach *et al.* (1988).

A study by Withey *et al.* (1983) of the absorption kinetics of four chlorinated organic compounds administered by a single gavage dose to rats in aqueous and corn oil vehicles demonstrated that, over a 5-hr observation period, a corn oil vehicle decreases the rate and extent of uptake of these compounds relative to that observed using an aqueous vehicle; the greatest decrease in uptake observed was for trichloroethylene, the least water-soluble compound tested. In the present analysis, oral absorption kinetics for CCl_4 were presumed to be similar to those observed by Withey *et al.* (1983) for trichloroethylene administered in 10 ml/kg of corn oil to 400-g Wistar rats, which yielded a roughly constant, 90-min absorptive pulse equivalent to a clearance of compound from the vehicle at a rate of about 0.11 ml vehicle cleared/kg body wt/min, resulting in a total area under the blood concentration times time curve (or AUBC, here assumed to be proportional to total absorbed dose) of about one 170th of that obtained using an aqueous vehicle (here assumed to reflect 100% uptake of the administered dose). This vehicle clearance or ingestive uptake rate (times 170^{-1}) was assumed to scale to the −0.26 power of body weight (Bogen, 1988) and to apply only to single-dose (and not to chronic) exposure scenarios involving a corn oil vehicle. The effect of such a vehicle on absorption efficiency of chronically administered oral doses of CCl_4 and similar compounds is currently unknown.

$CHCl_3$. Chloroform is metabolized in the liver to a reactive intermediate (phosgene) which is the proximate cause of this compound's hepatotoxicity (Ilett *et al.*, 1973; Brown *et al.*, 1974a; Lavigne and Marchand, 1974; McMartin *et al.*, 1981; U.S. EPA, 1985a). To estimate effective (metabolized) dose as a function of applied dose, a model of $CHCl_3$ metabolism was used based on the following $CHCl_3$-specific parameterization of the PBPK model of Ramsey and Andersen (1984). Tissue/air and blood/air partition coefficients used for mice were assumed to be equal to those obtained for rats and that obtained for mice, respectively, by Gargas *et al.* (1988, 1989) by *in vitro* techniques. Physiological parameter values used for mice and humans were the reference values cited above (U.S. EPA, 1988), except that the alveolar ventilation rate for 70-kg humans was increased to 378 liters/hr, based on the data reported in U.S. EPA (1988). The blood/air partition coefficient for humans was taken to be 8.0, the median of several reported values (Steward *et al.*, 1973; Sato and Nakajima, 1979; Gargas *et al.*, 1989). Human tissue/blood partition coefficients were similarly taken from the literature: 2.1 for liver and 35 for fat (Steward *et al.*, 1973), 1.9 for the richly perfused compartment (Feingold and Hola-

day, 1977), and 1.5 for the muscle/skin compartment (average of values for "muscle" and "vessel-poor" compartments reported by Feingold and Holaday, 1977). The Michaelis constant for mice and humans was set equal to 0.25 mg/liter, a value measured for rats (Gargas *et al.*, 1988).

Uptake of single doses of ingested $CHCl_3$ in corn oil by rats was observed by Withey *et al.* (1983) to yield a roughly constant pulse of absorption into blood lasting about 30 min. This corresponds to a rate of uptake from vehicle of 0.33 ml vehicle cleared/kg body wt/min, which was assumed in the present analysis to scale to the −0.26 power of body weight and to apply only to single-dose exposures involving a corn oil vehicle. Mass–balance metabolism studies of $CHCl_3$ orally administered in corn oil to mice and rats have shown that the majority of such a dose is metabolized to CO_2 within a period of hours after administration (Reynolds *et al.*, 1984; Mink *et al.*, 1986). Accordingly, it was assumed that mice absorb 100% of orally applied doses of CCl_4 in corn oil, and that, as with CCl_4, the total amount of $CHCl_3$ metabolized in 1 hr postexposure is the appropriate measure of the hepatotoxic effective dose.

V_{max} for 25-g mice (4.7 mg/hr) was estimated by fitting the metabolized dose predicted by the PBPK model, parameterized as stated except for V_{max}, to the average of the metabolized amounts observed for mice given a single gavage dose of 60 mg/kg CCl_4 in corn oil (Brown *et al.*, 1974b; Taylor *et al.*, 1974). V_{max} for a reference 70-kg human (560 mg/hr) was similarly estimated using data on metabolism, collected over 8 hr, from human volunteers given single oral doses of 500 mg CCl_4 in olive oil (Fry *et al.*, 1972). PBPK parameter values for mice and humans with different weights were scaled as described above.

CH_2Cl_2. Metabolism of this compound yields reactive species through both a saturable (oxidative mitochondrial) mixed-function oxidase pathway and a relatively nonsaturable (nonoxidative cytosolic) glutathione *S*-transferase (GST) pathway (Kubic *et al.*, 1974; Ahmed *et al.*, 1980; Gargas *et al.*, 1986b; Reitz *et al.*, 1986). These reactive metabolites, as those from other multihalogenated methanes, are likely to be responsible for exposure-related hepatotoxicity because the parent compound is relatively nonreactive with tissue macromolecules (Ahmed *et al.*, 1980). Consistent with this hypothesis is the similarity between hepatic cytoxicity observed in mice given acute or subchronic doses of CH_2Cl_2 and that observed in mice treated with CCl_4 (Weinstein *et al.*, 1972). In the present analysis, hepatotoxicity was presumed to arise only from that fraction of daily applied dose metabolized in liver by the GST pathway, which is consistent with the observed pattern of experimental CH_2Cl_2-induced mutagenicity in bacteria and tumorigenicity in rodents (Green *et al.*, 1988; Andersen *et al.*, 1987; U.S. EPA, 1987). To estimate this effective dose as a function of applied dose, the Reitz *et al.* (1988, 1989) parameterization of the PBPK model of Andersen *et al.* (1987) for CH_2Cl_2 in mice and humans was used. PBPK parameter values for mice and humans with different weights were scaled as previously described.

Model for Induced Cell Proliferation

Dose–response data on induced hepatocellular proliferation in mice for the three compounds considered were obtained from the studies listed in Table 2. To make use of the oral exposure studies involving $CHCl_3$ and CCl_4, oral uptake efficiency was estimated as described above. In the case of CCl_4, different groups of the 30-g mice used in the Doolittle *et al.* (1987) study were given single oral doses of from 0 to 100 mg/kg CCl_4 in 10 ml/kg corn oil for different study periods ranging from 1 to 14 days. For the present analysis, metabolized doses, D, corresponding to these applied daily doses were estimated as 1/170th of the ingested CCl_4, cleared from the vehicle at a constant rate of 0.22 ml/kg/min for 45 min, and injected into the liver compartment. Similarly, it was assumed for the present analysis that the oral doses of chloroform in the Reitz *et al.* (1982) study were administered to 30-g mice in 10 ml/kg corn oil vehicle, such that 100% uptake in the dosed mice occurred by constant infusion into the liver compartment over a 15-min period. The question of oral uptake efficiency does not arise in the case of the LeFevre and Ashby (1989) study of hepatocellular cell proliferation induced by CH_2Cl_2 delivered by inhalation.

The relationship between estimated values of acute effective dose D and corresponding predicted values of the percentage of hepatocytes in S-phase (%S) was modeled as a threshold-like relationship based on: (1) the obvious nonlinearity of the corresponding dose–response data for $CHCl_3$ and CCl_4, the two compounds for which sufficient relevant data were available to make such a direct determination (see Table 2, Fig. 1); (2) a specific assumption made here that such proliferation represents regenerative cell growth following necrosis caused by chemically induced cytotoxicity; and (3) the traditional use of threshold models for virtually all endpoints other than cancer and mutation in both descriptive and regulatory toxicology. Clearly, apparent nonlinearity in observed dose–response data can never disprove a linear dose–response relationship at very low doses for which data are not available. Furthermore, the choice of a threshold-like model is critical in the present analysis, because the use of a nonthreshold relationship (such as one including a linear function of effective dose) here would result in dramatically higher CKM-derived estimates of predicted increased risk over background associated with exposure to low doses (see Discussion). Therefore, the rationale for using a threshold-type dose response for in-

TABLE 2

LOGNORMAL MODEL OF HEPATOCELLULAR PROLIFERATION IN MICE EXPOSED TO CHLORINATED METHANES

Compound	Data source: strain: sex: weight	Assay type[a]	Applied dose[b]	Estimated effective dose[c], D (mg/kg liver)	$\%S$ (%)	Estimated parameters of dose–response model[d] μ (g/kg liver)	σ	ρ
CCl_4	Doolittle *et al.* (1987): CD-1: M: 30 g	$\%S$, vtr	0 (6)		0.5			
			1 (6)	0	3.0			
			2[e] (3)	0.046	1.0			
			10 (6)	0.092	1.0			
			20[e] (3)	0.45	2.0			
			25 (6)	1.1	2.0	0.0017	1.35	1.00
			30 (6)	1.3	5.5			
			40 (6)	1.7	19			
			50 (6)	2.1	22.5			
			50 (3)	2.1	30			
			100[e] (3)	3.9	34			
			100 (3)	3.9	27			
$CHCl_3$	Reitz *et al.* (1982): B6C3F1: M: 30 g[f]	^{3}H–DNA, viv	0 (5)	0	0.5[g]			
			15 (5)	271	0.5	4.03	2.02	0.20
			60 (6)	937	1.14			
			240 (6)	2240	7.23			
CH_2Cl_2	Lefevre and Ashby (1989): B6C3F1: M: 26 g	$\%S$, viv	0 (5)	0	0.026	5.79	2.02[h]	0.0131
			4000 (5)	902	0.162			

[a] $\%S$, determination of percentage of hepatocytes in S-phase ($\%S$) isolated 48 (CCl_4) or 24 (CH_2Cl_2) hr after oral administration of compound or vehicle alone; vtr, *in vitro* treatment of hepatocytes with [^{3}H]thymidine (^{3}H-T) after isolation by liver perfusion; viv, ip injection of animals with ^{3}H-T prior to isolation of hepatocytes by liver perfusion. ^{3}H-DNA, cellular regeneration index estimated by determining relative incorporation of ^{3}H-T in liver DNA. See corresponding references for details.

[b] Dose in mg/kg orally administered in corn oil vehicle (CCl_4, $CHCl_3$) or in ppm administered in respired air (CH_2Cl_2); number of animals used is shown in parentheses.

[c] Toxicologically effective dose to hepatocytes (total metabolized dose 1-hr postexposure for CCl_4 and $CHCl_3$, total GST-metabolized dose to liver for CH_2Cl_2) was estimated using physiologically based pharmacokinetic models described in the text.

[d] See text for explanation of lognormal dose–response model and its parameters. $\%S_0$, $\%S$ for $D = 0$. Values for μ and σ are least-squares estimates based on the model fit to the corresponding data; values for ρ are based on $\%S_0$ and corresponding values of effective bioassay dose d from Table 1, as explained in the text.

[e] Three animals received the dose at Time 0 and 24 hr (total of two doses).

[f] Assumed weight (actual value unspecified).

[g] Data not reported as $\%S$, but rather as ^{3}H-T in DNA. $\%S_0$ assumed to be equal to the value pertaining to CCl_4; other $\%S$ values were assumed to be equal to $\%S_0$ times the ratio of observed levels of ^{3}H-T in DNA of dosed animals to that in controls.

[h] Insufficient data for estimation of both μ and σ; σ was assumed to be equal to the value pertaining to $CHCl_3$.

duced hepatocellular proliferation will be discussed in detail.

The modeling choice that was made here reflects the difference in probable causal mechanism between induced cell proliferation and toxic endpoints that might reasonably be associated with a linear dose-response at low doses. The discrete, quantal, and potentially irreversible nature of DNA mutation caused by exposure to certain reactive chemicals or ionizing radiations is both theoretically and experimentally associated with a linear nonthreshold dose–response relationship at very low doses (NAS, 1980; NCRP, 1989). Indeed, this fact has been the basis for the traditional application of linear nonthreshold models in risk prediction for environmental carcinogens (U.S. EPA, 1980; Albert, 1981) and is the basis for the present application of a linear function of effective dose in the mutation component of the CKM models considered here (discussed below).

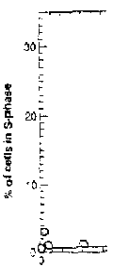

FIG. 1. Log
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more, even 10

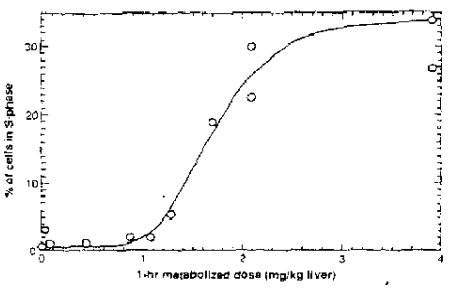

FIG. 1. Lognormal model (see Table 2 and corresponding text) fit to the data of Doolittle *et al.* (1987) on the percentage of hepatocytes in S-phase (%*S*) in male CD-1 mice 48 hr after exposure to different doses of CCl_4 in corn oil, administered orally either once or twice separated by 24 hr (see Table 2).

It is certainly conceivable that a single molecular interaction might be capable of causing cell replication either directly, or indirectly by causing cell death triggering compensatory proliferation, in a linear or "one-hit" fashion. For example, this may describe the action of a single hormone or growth factor molecule interacting with a highly specific cell-surface receptor molecule that functions as a highly sensitive signal transducer. Although it is difficult to imagine how this type of response might also apply to relatively nondistinct molecules like chloromethanes or their reactive metabolites, the possibility is suggested by the ability of CCl_4, $CHCl_3$, and CH_2Cl_2 to act as activators of protein kinase C in a cell-free *in vitro* system (Roghani *et al.*, 1987) and by the mitogenic activity of even very small concentrations of CCl_4 to human embryonic lung fibroblasts growing in culture (Brunius, 1987). However, results obtained with cell-free systems and *in vitro* systems involving proliferating embryonic cells may have no relevance to the proliferative behavior of adult cells *in vivo*, one reason being that three-dimensional intercellular interaction is likely to play an important regulatory role in adult nonregenerating tissue.

The chemical induction of cell death with definitively or approximately single-hit kinetics is known to occur, but it generally involves DNA damage resulting in a lethal mitotic event due to replication prior to repair of that damage (i.e., repair that is either total or sufficient to allow replication to proceed). This has been demonstrated for benzo[a]pyrene in cultured mouse liver epithelial cells (Landolph *et al.*, 1976) and for a number of alkylating agents and DNA-crosslinking agents in bacteria and DNA repair-deficient CHO cells (Thompson *et al.*, 1983; Hoy *et al.*, 1985). The compounds CCl_4, $CHCl_3$, and CH_2Cl_2, however, are only very weakly genotoxic, if at all, to mammalian cells *in vivo*. Furthermore, even 10-week exposures of rats to the potent alkylating agent diethylnitrosamine failed to induce (apparently necrosis related) hepatocellular proliferation in strict proportion to the administered dose; rather, induced replication was found to have a quite nonlinear dose response (Deal *et al.*, 1989). Extremely efficiently induced cell death, suggestive of single-hit kinetics apparently not mediated by DNA damage but rather by damage to the plasma membrane, has been observed in chemical- and radiation-exposed immature primordial oocytes from rodents and (even more dramatically so) from squirrel monkeys, but this greatly enhanced sensitivity is absent in growing or mature oocytes (Dobson and Felton, 1983). To the extent that this type of ultrasensitive response is known to involve only highly specialized immature cells, it would appear not to be relevant to hepatocytes under a lifetime, chronic exposure scenario.

There is currently no evidence that CCl_4, $CHCl_3$, or CH_2Cl_2 can exert a proliferative effect on hepatocytes (or any other cell type) at extremely low doses *in vivo*. On the contrary, CCl_4 and $CHCl_3$ are agents classically used to induce experimental hepatocellular necrosis and subsequent regenerative hyperplasia (Leevy *et al.*, 1959; Klassen and Plaa, 1966; Reynolds, 1967; Schultze *et al.*, 1973; Moore *et al.*, 1982). Perhaps the most is known regarding the mechanism of CCl_4-induced hepatocellular toxicity. The events preceding CCl_4-induced cell death are believed at present to involve the creation of a reactive trichloromethyl free-radical metabolite predominantly within the endoplasmic reticulum (ER) by the action of microsomal cytochrome P450 enzymes—but also by P450 activation at other cellular sites and by nonenzymatic activation by the electron transport chain within mitochondria—followed by secondary reactions of this metabolite with proteins and phospholipids that then interfere with macromolecule synthesis and function within the cell (Cheeseman *et al.*, 1985; Tomasi *et al.*, 1987; Villarruel *et al.*, 1987). One critical step in this process is now thought to be a resulting rapid inhibition of the ER CA^{2+} pump, leading to leakage of sequestered CA^{2+} from the ER and, if severe enough, to a postulated lethal disruption in intracellular calcium ion concentrations (Moore, 1980; Long and Moore, 1986; Ray and Moore, 1986). It is interesting in this regard that altered ER Ca^{2+} pump activity was not found to be linearly related to cytosolic CA^{2+} concentration and that the latter was highly nonlinearly related to applied CCl_4 (and even more so for applied $CHCl_3$) concentrations in studies using primary cultures of rat hepatocytes (Long and Moore, 1986). In particular, this study showed that while ER CA^{2+} pump activity, 5 min after exposure of cells to 0.3 mM CCl_4, underwent a significant decrease to two-thirds of that of nonexposed cells, the cytosolic Ca^{2+} concentration (as measured by phosphorylase *a* activity) was not significantly increased, indicating that a quite sizeable perturbation in ER Ca^{2+} pump activity was neces-

sary to disturb homeostasis in cytosolic Ca^{2+} concentration in the cells studied.

It is, then, apparently unlikely that a single event at a single target mediates the type of CCl_4-induced cell death that is known to stimulate hepatocellular proliferation; rather, in this case it is likely that the conjunction of multiple events at some point constitutes a level of irreversible cell injury that inevitably leads to eventual cell death (Smuckler and James, 1984). Although probably multiple, such events might nevertheless all impinge on a single most critical cell function affected (possibly, e.g., the maintenance of Ca^{2+} homeostasis by the combined activity of ER Ca^{2+} pump enzymes). Whether single or multiple, the disfunctions that mediate CCl_4-induced cell death are likely to involve, at some stage, the saturation of one or more critical enzymes. Enzyme saturation can explain threshold-like dose–response phenomena involving even the simplest types of chemical reaction (Licko, 1972). The assumption that a given response, such as death of an individual animal or of an individual cell, requires the joint occurrence or interaction of exceedances of many such thresholds, and that these thresholds have a normal (Gaussian) distribution with respect to dose, gives rise to the lognormal (or "logprobit") dose–response model, which is one of the most widely used models in classical toxicology and pharmacology for the description and analysis of dichotomous quantal dose–response data (Finney, 1952; Levine, 1983).

For the reasons discussed above, the relationship between estimated values of acute effective dose D and corresponding predicted values of the percentage of hepatocytes in S-phase ($\%S$) was modeled using the lognormal dose–response model.

$$\%S_D = \frac{100\%}{3}\Phi\left\{\frac{\log(D/\mu)}{\log(\sigma)}\right\} + \%S_0, \qquad (1)$$

in which Φ is the cumulative normal distribution function, $\%S_0$ is the background level of $\%S$ (i.e., at $D = 0$), μ is the location parameter (the dose corresponding to the median or geometric mean response), and σ is the shape parameter (the geometric standard deviation of the lognormally modeled response). The factor of 100%/3 and the corresponding assumed upper bound of (33% + $\%S_0$) for $\%S$ used in Eq. (1) account for the fact that $\%S$ represents a fraction of the total hepatocytes undergoing replicative DNA synthesis at 24 or 48 hr postexposure, whereas not all hepatocytes induced into S-phase are involved in synthesis precisely at that time. Clearly, a synchronous induction of 100% of hepatocytes into S-phase would be lethal. Detailed experiments have shown that CCl_4 intoxication or partial hepatectomy in rats and mice induces a wave of regenerative hepatocellular proliferation corresponding to an S-phase pulse commencing between 24 and 48 hr after administration and persisting between approximately 24 and 36 hr (Leevy *et al.*, 1959; Gerhard, 1973; Rabes *et al.*, 1973; Schultze *et al.*, 1973). Such proliferative response kinetics have been shown to be quite heterogeneous within rat liver, with hepatocytes in most liver zones reaching maximum $\%S$ values of between 20 and 35% at various times after induced acute liver injury (Gerhard, 1973; Rabes *et al.*, 1973). Furthermore, the average duration of S-phase in rats is about 8 hr (Rabes, 1973), which is about one-third of the average (24-hr) duration of dramatically increased S-phase activity after chemically or physically induced hepatocellular trauma (Leevy *et al.*, 1959; Gerhard, 1973; Rabes *et al.*, 1973; Schultze *et al.*, 1973). This suggests that 1- or 2-day interruptions in a chronic oral dosing regimen, such as the 5-day/week oral dosing regimen typical in cancer bioassays, would not substantially change the time-weighted average value of $\%S$ associated with that regimen in comparison with that of an uninterrupted regimen. A nonresponse in average $\%S$ to such an interrupted dosing regimen was therefore assumed in the present analysis for the compounds (CCl_4, $CHCl_3$) considered here that were orally administered in bioassays on a 5-day/week schedule.

The lognormal model described above was fit to the data shown in Table 2 using least-squares optimization of values for μ and σ, yielding the best-fit values listed in that table. (Confidence bounds for the lognormal parameter estimates listed in Table 2 are omitted—see Discussion.) The fit of the model to data on induction of $\%S$ in hepatocytes of mice orally dosed (either once, or twice separated by 24 hr) with CCl_4 is shown in Fig. 1.

The Doolittle *et al.* (1987) study included some investigation of how chronic (daily) dosing affects $\%S$ and serum enzyme levels in mice as compared to single dosing. Their results indicate that induced $\%S$ is lower with chronic as opposed to single CCl_4 dosing by a factor of 2 to 3. For the present analysis, it was assumed for all compounds considered that the factor 100%/3 in Eq. (1) must be replaced by 100%/9 when this model is applied to chronic dosing situations. The latter assumption implies that no chronic effective dose can produce a value of $\%S$ greater than $\%S_{max}$, where

$$\%S_{max} = \frac{100\%}{9} + \%S_0. \qquad (2)$$

Using this model, the predicted value of $\%S_{max}$ equals 11.6% for the Doolittle *et al.* (1987) study.

CKM Models Applied to Liver Cancer Data for Mice

A mathematical description of the approximations for the two-stage exponential and geometric CKM models used in the present analysis is given by Bogen (1989). These models predict the expected value of cumulative cancer risk, R, as a function of age (minus a latency period here assumed to be 0) for a constant, lifetime expo-

TABLE 3

FITTED VALUES FOR GROWTH AND MUTAGENIC POTENCY PARAMETERS IN CKM MODELS[a] OF LIVER TUMORIGENICITY OF CHLORINATED METHANES TO MICE

		CKM model type[a]					
		Exponential			Geometric		
Compound	Assumed tumorigenic mechanism	$g_1 = g_3$ (year^{-1})	g_2 (year^{-1})	b (μg/kg liver)$^{-1}$	$a_1 = a_3$ (year^{-1})	a_2 (year^{-1})	b (μg/kg liver)$^{-1}$
CCl_4	Promotion	1.91	>6.54	0	0.939	>12.7	0
	Mutation	1.91	1.91	>1140	0.939	0.939	>1090
$CHCl_3$	Promotion	1.91	6.18	0	0.939	10.6	0
	Mutation	1.91	1.91	8.19	0.939	0.939	7.79
CH_2Cl_2	Promotion	2.51	3.80	0	1.63	3.93	0
	Mutation	2.51	2.51	1.74	1.63	1.63	1.62

[a] See text for explanation of CKM dose–response models and their estimated parameters. The numerical subscripts i on the growth parameters, g_i and a_i, $i = 1, 2, 3$, refer to corresponding exposure endpoints t_i, where exposure to effective dose d is presumed to occur only during time t for $t_1 \leq t \leq t_2$. The parameter values listed are exact fits to dose–response data corresponding to control and low-dose bioassay responses, R_0 and R_d, respectively (see Table 1).

sure scenario, using the relations given by Eqs. (E-4) and (G-3) in the Appendix. To account for exposure scenarios involving n distinct exposure periods (i.e., in cases involving a sequence of n sets of different parameter values governing mutation and/or cell proliferation rates), the latter equations must be replaced by Eqs. (E-7) and (G-6), respectively, from the Appendix. All of the bioassays considered here involved preexposure, exposure, and postexposure periods. Therefore, to model hepatocellular cancer risk to bioassay mice, Eqs. (E-7) and (G-6) were used with n set equal to 3 and with x (the exponent in the geometric CKM model) in Eq. (G-6) set equal to 3. The three distinct exposure periods ending at times t_i, $i = 1, 2, 3$, respectively, used in each bioassay considered are shown in Table 1.

To fit the CKM models, it was assumed that bioassay mice have 1.5×10^8 hepatocytes/g liver tissue (Peterson, 1985) at risk throughout their lives, of which 25% are diploid cells at risk for malignant transformation (Danielsen, 1988). Assuming liver is 5.5% of mouse body weight (U.S. EPA, 1988), the total number, N, of cells at risk is equal to body weight times 2×10^6 target cells/g body wt. It was further assumed that for nondosed animals the first and the second interstage mutation rates, $m_1 = m_3$ and $M_1 = M_3$ (see Appendix), are all equal to 5×10^{-6} y^{-1} (Bogen, 1989). The value of $K_0 = (N)(m_1)(M_1)$ for each bioassay data set considered is listed in Table 1.

Next, the cell growth parameters of the exponential and geometric CKM models, g and a, respectively (see Appendix), were fit (exactly) to the data pairs (R_0, R_d) given in Table 1 for each bioassay under the assumption that the corresponding compound is a "pure promoter" within the CKM framework described above. Specifically, in this first analysis, each of the compounds was assumed to act only to increase the proliferation rate of cells in "initiated" or premalignant (stage-1) clones that are themselves assumed to be resistant to the cytotoxicity of necrogenic doses but nevertheless stimulated to proliferate to the same extent as are surrounding normal hepatocytes during regenerative growth following dosing, which is consistent with experimental data on the comparative proliferative behavior of cells in rodent hepatocellular foci and surrounding normal cells (Dragani *et al.*, 1986). Here, d is the PBPK estimate of the lowest effective bioassay dose (also listed in Table 1) to liver tissue at which a significantly increased liver tumor incidence was observed, R_0 is background tumor risk, and R_d is observed tumor risk at effective dose d. Under the pure promoter assumption, the somatic mutation rates during dosing, m_2 and M_2, are equal to those for nondosed animals. For each compound, two unique values were obtained for the growth parameters g and a corresponding to nondosed periods ($g_1 = g_3$ and $a_1 = a_3$, respectively) and the dosing period (g_2 and a_2, respectively) of the bioassay serving as the basis for risk extrapolation. The values obtained are listed in Table 3. The corresponding exponential and geometric CKM models fit to the two (control and low-dose) bioassay data points for CCl_4 are shown in Fig. 2a.

To proceed with the "promotion-only" analyses, it was assumed that an increase in $\%S$ over $\%S_0$ by a given

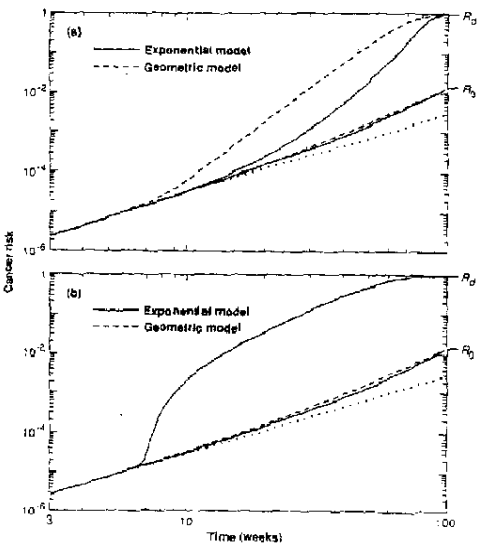

FIG. 2. Two-stage cell-kinetic multistage (CKM) models (see Table 3 and corresponding text) fit to control and low-dose bioassay data (see Table 1) on CCl_4-induced hepatocellular carcinoma incidence in female B6C3F1 mice. Dotted lines represent hypothetical CKM-predicted probability of tumor occurrence in the absence of proliferation of initiated (premalignant) cells. (a) Promotion-only CKM models; (b) mutation-only CKM models; exponential and geometric models approximately coincide for dosed animals.

fraction f of its predicted (or measured) bioassay-associated range, induced by a given effective dose D, causes a directly proportional increase in the net growth rate of premalignant cells. That is, it was assumed that

$$f = s\frac{\%S_D - \%S_0}{\%S_d - \%S_0} = \frac{z_D - z_0}{z_d - z_0}, \qquad (3)$$

where

$$0 \leq D \leq d$$

$$\%S_0 \leq \%S_D \leq \%S_d \leq \%S_{\max}$$

$$0 < s \leq 1$$

$$z_0 \leq z_D \leq z_d$$

$$0 \leq f \leq 1,$$

where z is a CKM growth parameter equal to either g or a; the D subscript refers to a value corresponding to the specified effective dose D; and the d and 0 subscripts refer to values pertaining to dosed and control bioassay animals, respectively. The upper bound of 1 on s given above accounts for the fact that $\%S$ is an indication only of cell replication rate, not of cell death rate. An enhanced replication rate in proliferating premalignant cells that is *also* associated with an enhanced cell death rate would lead to values of s less than 1. For the present analysis, in the absence of information on dose-response for compound-induced changes in cell death rates *in vivo*, it was assumed that $s = 1$.

Analyses were next performed under the contrasting assumption that each of the compounds considered is only mutagenic and has no effect on premalignant cell proliferation. In these analyses, the first and second mutation rates during dosing, m_2 and M_2, were assumed to be equal linear functions of effective dose D, given by

$$m_2 = M_2 = m_0(1 + bD), \qquad (4)$$

in which m_0 represents the spontaneous mutation rate ($m_1 = m_3 = M_1 = M_3$) and b represents the relative mutagenic potency of effective dose D. Under this assumption, it follows that $g_1 = g_2 = g_3$ and $a_1 = a_2 = a_3$ for each compound, and the corresponding calculated values of b are given in Table 3. (For example, the corresponding exponential and geometric CKM models fit to the bioassay data for CCl_4 are shown in Fig. 2b.) To proceed with the "mutation-only" analyses, the fraction f was defined simply as the ratio D/d for $0 \leq D \leq d$.

Finally, in all analyses, background risk R_0 was assumed to be independent such that increased risk was defined as $A = (R - R_0)/(1 - R_0)$, where $R_0 \leq R \leq R_d$ and the d and 0 subscripts again refer to values pertaining to dosed and control bioassay animals, respectively.

The fitted CKM models, along with the assumptions stated above, were used to numerically calculate f as a function of A for each of the models and compounds considered. In each case, the particular, unique value of f, denoted f^*, was calculated to correspond to a predicted increased risk of $A = 10^{-6}$. These values are listed in Table 4. Next, the relations between D and f for the promotion-only and mutation-only CKM models were used to calculate the virtually safe effective dose D^* corresponding to f^* defined above. For the mutation-only analyses, the relation is simply $D^* = (f^*)(d)$. For the promotion-only analyses, the corresponding relation follows from Eq. (1), appropriately modified to reflect a chronic dosing assumption, and from Eq. (2), which together imply that

$$\begin{aligned} D &= \mu\sigma^{\Phi^{-1}\{(9/100\%)(\%S_d - \%S_0)\}} \\ &= \mu\sigma^{\Phi^{-1}\{(9/100\%)(f/s)(\%S_d - \%S_0)\}} \\ &= \mu\sigma^{\Phi^{-1}\{(f/s)(\%S_d - \%S_0)/(\%S_{\max} - \%S_0)\}}. \end{aligned} \qquad (5)$$

For convenience, the magnitude of $\%S_d$ relative to $\%S_{\max}$, defined in Eq. (2) by shall be described using the ratio ρ defined as

TABLE 4

VIRTUAL SAFE DOSE[a] OF CHLORINATED METHANES EXTRAPOLATED FROM BIOASSAY DATA ON MOUSE LIVER TUMORS

Compound	Assumed tumorigenic mechanism[d]	Exponential CKM model[b] f^* ($\times 10^{-6}$)	Exponential CKM model[b] D^* (mg/kg liver)	Exponential CKM model[b] VSD (mg/day)	Geometric CKM model[b] f^* ($\times 10^{-6}$)	Geometric CKM model[b] D^* (mg/kg liver)	Geometric CKM model[b] VSD (mg/day)	Linearized multistage model[c] VSD (μg/day)
CCl_4	P	>18	<0.49	<3.5	>5.0	<0.45	<3.0	—
	M	>2.4	<5.2 $\times 10^{-5}$	<2.5 $\times 10^{-4}$	>2.4	<5.2 $\times 10^{-5}$	<2.5 $\times 10^{-4}$	0.95
$CHCl_3$	P	20	170	950	6.3	150	730	—
	M	3.2	0.0071	0.011	3.2	0.0071	0.011	0.30
CH_2Cl_2	P	9.0	153	9500	6.1	145	9100	—
	M	5.0	0.0060	2.7	5.0	0.0060	2.7	23

[a] Here, the virtual safe dose (VSD) is defined as that daily dose ingested by a 60-kg human over a lifetime associated with a predicted cancer risk of 10^{-6}.

[b] See text for explanation of CKM dose–response models and their estimated parameters.

[c] VSDs listed for CCl_4, $CHCl_3$, and CH_2Cl_2 were calculated using the corresponding 95% upper-bound carcinogenic potencies of 0.063, 0.20, and 0.0026 $(mg/kg/day)^{-1}$, respectively, derived by the U.S. Environmental Protection Agency (U.S. EPA, 1984, 1985a,b) using the "linearized" multistage dose–response extrapolation model (described in those references) applied to data on hepatocellular carcinoma induction in female B6C3F1 mice from all dose groups in the bioassays considered in the present study (see Table 2). The potency derived for CCl_4 was based on pooled data for male and female mice; that for CH_2Cl_2 was based on data on incidence of hepatocellular carcinoma or adenoma. Metabolism was considered in potency estimation only in the case of $CHCl_3$, where 94% metabolism of the administered bioassay doses were assumed (U.S. EPA, 1985a).

[d] P, promotion only; M, mutation only.

$$p = \frac{\%S_d - \%S_0}{\%S_{max} - \%S_0} = \Phi\left\{\frac{\log(d/a)}{\log(b)}\right\}, \qquad (6)$$

whereupon it follows from Eqs. (3), (5), and (6) that

$$D = \mu\sigma^{\Phi^{-1}\{p/s\}}. \qquad (7)$$

For the promotion-only analyses, then, D^* was calculated using Eq. (7) in which f^* was substituted for f, where it was assumed that $s = 1$ (see discussion above) and where the values for μ, σ, and ρ are listed in Table 2.

Extrapolation of Liver Cancer Risk to Humans

It was assumed that the virtually safe effective dose obtained for female bioassay mice applies to humans as well, i.e., that human hepatocytes are as equally susceptible to compound-mediated enhanced proliferation as mouse hepatocytes. To complete the CKM-based risk analysis, PBPK models for reference 60-kg (females) humans were used with parameter values obtained as discussed above to estimate a human daily effective dose, D, as a function of a chronic ingested dose (e.g., daily uptake from drinking water) for each of the compounds considered. Exposures were presumed to occur once per day, each modeled to result in a constant 6-min infusion of 100% of ingested dose into the liver compartment, and simulated daily exposures were repeated (for 7 to 10 simulated exposure days) until virtual dynamic equilibrium in daily effective dose was attained.

RESULTS

Best-fit parameter values obtained for the lognormal models fit to data on induced cell proliferation for each of the compounds are shown in Table 2. The promotion-only and mutation-only CKM models fit to mouse bioassay data on hepatocarcinogenicity for each of the comopunds are summarized in Table 3. Based on the information from Tables 2 and 3, as explained under Methods, calculated values of a virtually safe effective

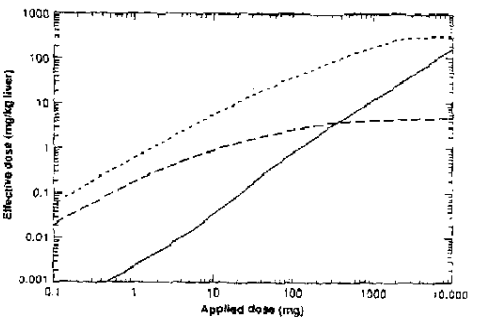

FIG. 3. Hepatotoxic effective dose to liver tissue of a 60-kg human (reference female) chronically exposed to a daily single ingested dose of CCl_4 (medium-dashed curve), $CHCl_3$ (short-dashed curve), or CH_2Cl_2 (solid curve), as predicted by physiologically based pharmacokinetic models described in the text. Effective dose was assumed to be total 1-hr metabolized dose postexposure (in mg/kg liver tissue) for CCl_4 and $CHCl_3$, whereas for CH_2Cl_2 it was assumed to be total daily dose (in mg/kg liver tissue) metabolized in liver by the glutathione *S*-transferase pathway.

dose, D^*, for the different compounds and CKM models are listed in Table 4.

The relations between chronic applied dose and a PBPK-predicted human effective dose for each of the compounds are shown in Fig. 3. This information was used to obtain a human applied VSD corresponding to each value of D^* listed in Table 4. These calculated VSDs for the different CKM models are shown in Table 4, where they are compared to VSDs for each compound calculated using the "linearized" multistage model applied to the same mouse bioassay data on induced hepatocarcinogenesis that served as the basis for CKM-based risk extrapolation.

DISCUSSION

Because the CKM-based approach to cancer risk assessment illustrated here for chlorinated methanes allows for a threshold-like dose response for agents considered to be "pure promoters," corresponding VSDs associated with exposure to such agents will typically be much lower than those based on nonthreshold risk-extrapolation methods that presume increased cancer risk for these compounds is due solely to increased mutation rates that are linear as a function of dose at low doses. Thus, in this analysis for chlorinated methanes, the VSDs calculated using promotion-only CKM models are between three and five orders of magnitude higher than those calculated using mutation-only CKM models and are from three to more than seven orders of magnitude higher than those calculated using the linearized multistage model. Using the promotion-only CKM models, the geometric model yielded slightly but consistently lower (more conservative) VSDs than the exponential one, as expected (Bogen 1989).

It must be emphasized that many uncertainties remain in defining the most appropriate way to implement CKM-based cancer risk extrapolation, even assuming the CKM approach is preferable. The most fundamental source of uncertainty lies in the assumption made regarding the extent to which the observed carcinogenicity of a given compound (e.g., in bioassay animals) is due to promotion (enhanced cell proliferation rates) alone, which in some cases might reasonably have a threshold-like dose–response relationship, and how much is due to increased mutation rates, which in two-stage CKM models have a linear–quadratic dose–response relationship. Clearly, if a compound is assumed to have any degree of mutagenicity at all, this effect will ensure that the CKM-predicted increased risk as a function of effective dose will be linear at sufficiently low doses (Portier, 1987). In particular, it is easily shown that if, within a CKM framework in which induced cell proliferation is assumed to have a threshold dose response, increased mutation actually accounts for P percentage of an observed increased tumor incidence at high doses, then increased risk calculated using a conservative mutation-only CKM model extrapolated to very low

doses will overestimate true risk by a factor of approximately $100\%/P$.

Some of the other sources of uncertainty in the foregoing CKM-based risk extrapolation for chlorinated methanes include:

(1) the effects of corn oil vehicle and chronic dosing on ingestive uptake of administered dose;

(2) the selection of the most appropriate (compound-specific) PBPK measure(s) of interspecies equitoxic effective dose for cytotoxic (cell-proliferative) and genotoxic effects on hepatocytes;

(3) the selection, assuming the validity of the CKM approach, of an appropriate form of CKM model to use, e.g., exponential vs geometric, semistochastic vs fully stochastic;

(4) the quantitative relationship between changes in measured increases in $\%S$ and changes in actual net proliferation (i.e., birth minus death) rates in premalignant cells;

(5) the very limited dose–response data available on increased $\%S$ in mouse liver induced by acute exposure, particularly for CH_2Cl_2 and in general for doses spanning a range large enough to validate a model (such as a lognormal model) for low-dose dose response;

(6) the relationship between increased $\%S$ induced by acute exposure to that induced by long-term chronic exposure similar to that experienced by bioassay animals;

(7) the statistical uncertainty omitted from the present analysis by failing to consider confidence bounds on estimated CKM parameter values and by using information on the tumor response of only the low-dose groups from the mouse bioassays to the exclusion of available data from higher dose groups; and

(8) the unknown dose response for liver cancer induction in mice receiving CCl_4 doses less than the low-dose level used in the NCI (1976a,b, 1977) cancer bioassays for that compound.

Some of these uncertainties can be reduced or eliminated by the acquisition of the appropriate experimental data (Nos. 1, 2, 4–6, and 8, above). In particular, there is a clear need to gather much more detailed data on proliferative response to chronic dosing, especially among cells within putative preneoplastic foci in animals receiving very low chronic dose levels. Defining rather precise dose–response relations for such induced proliferation should be experimentally achievable because it is possible to score a sufficiently large number of cells (from even a relatively small number of experimental animals) to obtain statistically reliable dose–response characterizations (particularly with automated cell scoring techniques). Another uncertainty source (No. 7) is fairly easily addressed by the incorporation of standard statistical procedures within the general approach to CKM-based cancer risk extrapolation outlined above. This was not undertaken in the present investigation because its focus was on analytic and methodological issues concerning the distinction between the mutation-only and promotion-only approaches, as plausible extremes, to CKM-based cancer risk prediction. (However, it is unlikely that the wide divergence in risks predicted here using these two approaches would have been greatly reduced had such statistical considerations been addressed.) Other sources of uncertainty (No. 3) are unlikely to be significantly reduced until a better understanding of spontaneous and chemically induced carcinogenesis is obtained.

Despite the difficulties that are involved in addressing such uncertainties, the CKM approach incorporates the emerging facts regarding carcinogenesis to a far greater extent than do the more traditional cancer risk extrapolation models. Even when data available to implement a CKM-based risk extrapolation for a given compound are limited, consideration of this quantitative approach may yield readily testable hypotheses. For example, if a suitable chronic dose level were

selected, the CKM initiator-only and promoter-only models for $CHCl_3$-induced hepatocarcinogenesis in mice discussed above would predict very different tumor incidences in 100 exposed bioassy mice compared to those in controls. In particular, if 30-g female B6C3F1 mice were administered a chronic daily dose of 67 mg/kg $CHCl_3$ by gavage, the PBPK model for this compound used earlier would predict a corresponding 1-hr effective dose of 1000 mg/kg liver tissue. This effective dose, in turn, would induce a predicted increase in the value of the cell growth parameter, in either the exponential or geometric CKM protomoter-only models, equal to 10% of that necessary to raise the incidence from its spontaneous rate of 1.25% to the rate of 88.9% observed in bioassay mice given a chronic oral dose of 230 mg/kg/day (NCI, 1976c; Reuber, 1979; see Table 1), according to the lognormal model of induced cell proliferation for $CHCl_3$ specified in Table 2. This proliferative response, in turn, would be predicted to result in an incidence rate of hepatocellular carcinoma between 1.9 and 3.7%, according to the CKM promoter-only models for $CHCl_3$ specified in Table 2. However, according to the corresponding mutation-only models, the incidence rate should be 41%. Time-to-tumor information from bioassays involving serial terminations would, of course, greatly improve the ability to test a promoter-only hypothesis within the CKM framework and, based on spontaneous tumor incidence data alone, might even be used to test exponential CKM models against their geometric counterparts.

Finally, it should be emphasized that even if the CKM framework accurately characterizes both spontaneous and chemically induced carcinogenesis, some—perhaps even most—compounds experimentally observed to be carcinogenic to animals are likely to cause this response by eliciting a mixture of mutagenic and cell-proliferative processes. There is some degree of correlation between mutagenicity and cytotoxicity (Grisham and Smith, 1984; Peterson and Peterson, 1985). It may therefore be that, as a consequence of associated cytotoxicity, some classically mutagenic carcinogens indirectly elicit a proliferative response sufficient to account for some or most of their observed carcinogenicity (within the CKM framework), as suggested by the recent data of Deal *et al.* (1989) on diethylnitrosamine-induced hepatocellular proliferation in rats. It may also be that carcinogenic compounds with little observed genotoxic activity in mammalian cells *in vivo*, such as CCl_4, may exert their carcinogenic effect solely by means of mutation (perhaps by some as yet unidentified mechanism). Because mutagenic potential can never be ruled out definitively, the most controversial issue involved in the application of CKM models to cancer risk assessment is likely to be the definition of the necessary elements of any data base sufficient to warrant a reasonable conclusion that a given compound is very unlikely to be genotoxic to mammalian cells *in vivo*, such that it might be considered a pure promoter within the CKM framework.

APPENDIX

For CKM models in general, the stage-j cell populations of a k-stage process of carcinogenesis are presented by the stochastic processes $X_j(T)$, $j = 1, \ldots, k$, over the period $0 \leq t \leq T$, where $X_j(0) = 0$; $X_0(T)$ is a deterministic nonnegative (DN) ("driving") function; and $m_j(T)$, for $j = 0, \ldots, k-1$ and $m_j(T)T \ll 1$, are DN functions (stage-specific mutation rates) such that the probability of a unit gain by $X_{j+1}(T)$ caused by a simultaneous unit loss from $X_j(T)$ during the interval $(T, T + dT)$ is approximately $m_j(T)dT$, and the probability of more than one such event occurring in this interval is negligible. In semistochastic CKM models, $w_j(T)$, $j = 1, \ldots, k-1$, are DN impulse-response (or weighting) functions used to represent (premalignant) cell proliferation subsequent

to the occurrence of each new stage-j clone arising by mutation of a stage-$(j-1)$ cell, where $w_k(T) = 1$. Finally, let $R(t) = \text{Prob}\{X_k(t) \geq 1\}$, the cumulative distribution of the waiting time for the occurrence by time t of the first increment in $X_k(t)$, representing the occurrence of the first cancer cell, which here is presumed to result rapidly in a lethal tumor. It has been shown (Bogen, 1989) for semistochastic CKM models in general that $R(t) \approx 1 - \exp(EX_k(t))$, where E denotes the expectation operator.

Below are the approximate solutions for two-stage (i.e., $k = 2$), semistochastic, exponential, and geometric CKM models of carcinogenesis, for the case where parameter values governing mutation rates and cell proliferation in intermediate-stage (here, in stage-1) cells change at times t_i for $i = 1, \ldots, n$, where $t_0 = 0$, $t_{i-1} \leq t_i$, and $t_n = t$. Here it is assumed that $X_0(T)$ is equal to the constant X_0 and that $m_j(t_{i-1} \leq T \leq t_i)$ for $j = 0$ and $j = 1$ are equal to the constants m_i and M_i, respectively. From these assumptions, it follows (Bogen, 1989) that for both exponential and geometric CKM models,

$$EX_1(t_n) \approx \sum_{i=1}^{n} \int_{t_{i-1}}^{t_i} X_0 m_i w_1(t_i - T)\,dT, \tag{A-1}$$

and

$$EX_2(t_n) \approx EX_2(t_{n-1}) + \int_{t_{n-1}}^{t_n} M_n EX_1(T)\,dT. \tag{A-2}$$

In the following evaluations of Eqs. (A-1) and (A-2), the growth response function, $w_1(T)$, for all proliferating premalignant (i.e., stage-1) cells is taken to be a function of the growth parameter z, where z is designated g and a in exponential and geometric CKM models, respectively, and where z at time T equals the constant z_i for $t_{i-1} \leq T \leq t_i$, $i = 1, \ldots, n$.

I. Exponential Two-Stage CKM Models

For two-stage exponential CKM models, we first define the functions

$$\hat{X}_1(t_i) = X_0 \frac{m_i}{g_i}(e^{g_i(t_i - t_{i-1})} - 1) \tag{E-1}$$

$$\hat{X}_2(t_i) = X_0 \frac{m_i M_i}{g_i^2}(e^{g_i(t_i - t_{i-1})} - g_i(t_i - t_{i-1}) - 1) \tag{E-2}$$

and note that, because $w_1(T) = e^{gT}$ when $n = 1$, Eqs. (A-1) and (A-2) imply that

$$EX_1(t_1) \approx \hat{X}_1(t_1), \tag{E-3}$$

and

$$EX_2(t_1) \approx \hat{X}_2(t_1). \tag{E-4}$$

To consider cases in which $n > 1$, we now define

$$\epsilon_{r,s} = \begin{cases} \sum_{i=r}^{s} g_i(t_i - t_{i-1}) & \text{for } r \leq s \\ 0 & \text{for } r > s \end{cases} \tag{E-5}$$

and note that $w_1(T) = \exp(g_i T + \epsilon_{i+1,n})$ during the period t_{n-1} to t_n for stage-1 clones arising at time T where $t_{i-1} \leq T \leq t_i$. Upon substitution and integration, Eqs. (A-1) and (A-2) yield

$$EX_1(t_n) \approx \sum_{i=1}^{n} \hat{X}_1(t_i)\exp(\epsilon_{i+1,n}), \tag{E-6}$$

and

$$EX_2(t_n) \approx \sum_{i=1}^{n} \hat{X}_2(t_i) + \sum_{i<j\leq n} \hat{X}_1(t_i)\hat{X}_1(t_j)X_0^{-1}\exp(\epsilon_{i+1,j-1}). \tag{E-7}$$

II. Geometric Two-Stage CKM Models

For two-stage geometric CKM models in the case where $n = 1$, it has been shown (Bogen, 1989) that $w_1(T) = (aT + 1)^x$ (where, e.g., $x = 3$ if premalignant clones are assumed to grow spherically with radial growth ultimately proportional to time). For these geometric CKM models, we first define the function

$$\dot{X}_2(t_i) = X_0 \frac{m_i M_i}{(x+1)(x+2)a_i^2} \times [(a_1(t_i - t_{i-1}) + 1)^{x+2} - (x+2)a_1(t_i - t_{i-1}) + 1] \quad \text{(G-1)}$$

and note that from Eqs. (A-1) and (A-2) it follows that

$$EX_1(t_1) \approx X_0 \frac{m_1}{(x+1)a_1}[(a_1 t_1 + 1)^{x+1} - 1], \quad \text{(G-2)}$$

and

$$EX_2(t_1) \approx \dot{X}_2(t_1). \quad \text{(G-3)}$$

To consider cases in which $n > 1$, we now define

$$\gamma_{r,s} = \begin{cases} 1 + \sum_{i=r}^{s} a_i(t_i - t_{i-1}) & \text{for } r \leqslant s \\ 1 & \text{for } r > s \end{cases} \quad \text{(G-4)}$$

and note that $w_1(T) = (a_i T + \gamma_{i+1,n})^x$ during the period t_{n-1} to t_n for stage-1 clones arising at time T where $t_{i-1} \leqslant T \leqslant t_i$. Upon substitution and integration, Eqs. (A-1) and (A-2) now yield

$$EX_1(t_n) \approx \sum_{i=1}^{n} X_0 \frac{m_i}{(x+1)a_i}(\gamma_{i,n}^{x+1} - \gamma_{i+1,n}^{x+1}), \quad \text{(G-5)}$$

and

$$EX_2(t_n) \approx \sum_{i=1}^{n} \dot{X}_2(t_i) + \sum_{i<j\leqslant n} \left[X_0 \frac{m_i M_j}{(x+1)(x+2)a_i a_j} \times (\gamma_{i,j}^{x+2} - \gamma_{i,j-1}^{x+2} - \gamma_{i+1,j}^{x+2} + \gamma_{i+1,j-1}^{x+2}) \right]. \quad \text{(G-6)}$$

ACKNOWLEDGMENTS

This work was performed under the auspices of the U.S. Department of Energy at the Lawrence Livermore National Laboratory under Contract W-7405-ENG-48, funded in part by the State of California, Department of Health Services (Contract 87-T0102), and the U.S. Environmental Protection Agency Office of Research and Development (Contract DW-8993-4205).

REFERENCES

AHMED, A. E., KUBIC, V. L., STEVENS, J. L., AND ANDERS, M. W. (1980). Halogenated methanes: Metabolism and toxicity. *Fed. Proc.* **39**, 3150–3155.

ALBERT, R. M. (1981). A biological basis for the linear non-threshold dose-response relationship for low-level carcinogen exposure. In *Measurement of Risks* (G. G. Berg and H. D. Maille, Eds.), pp. 535–541. Plenum, New York.

ANDERSEN, M. E., CLEWELL, H. J., III, GARGAS, M. L., SMITH, F. A., AND REITZ, R. L. (1987). Physiologically based pharmacokinetics and the risk assessment process for methylene chloride. *Toxicol. Appl. Pharmacol.* **87**, 185–205.

ANDRAE, U., AND WOLFF, T. (1983). Dichloromethane is not genotoxic in isolated rat hepatocytes. *Arch. Toxicol.* **52**, 287–290.

ARMITAGE, P., AND DOLL, R. (1957). A two-stage theory of carcinogenesis in relation to the age distribution of human cancer. *Brit. J. Cancer* **11**, 161–169.

ARMITAGE, P., AND DOLL, R. (1961). Stochastic models for carcinogenesis. In *Fourth Berkeley Symposium on Mathematics, Statistics, and Probability*, pp. 19–38. Univ. of California Press, Berkeley.

BERMUDEZ, E., MIRSALIS, J. C., AND HALES, H. C. (1982). Detection of DNA damage in primary cultures of rat hepatocytes following *in vivo* and *in vitro* exposure to genotoxic agents. *Environ. Mutagen.* **4**, 667–679.

BOGEN, K. T. (1988). Pharmacokinetics for regulatory risk analysis: The case of trichloroethylene. *Regul. Toxicol. Pharmacol.* **8**, 447–466.

BOGEN, K. T. (1989). Cell proliferation kinetics and multistage cancer risk models. *J. Natl. Cancer Inst.* **81**, 267–277.

BRIDGES, B. A., ZIEGER, E., AND MCGREGOR, D. B. (1981). Summary report on the performance of bacterial mutation assays. *Prog. Mutat. Res.* **1**, 49–67.

BROOKES, P., AND PRESTON, R. J. (1981). Summary report on the performance of *in vitro* mammalian assays. *Prog. Mutat. Res.* **1**, 77–85.

BROWN, D. M., LANGLEY, P. E., SMITH, D., AND TAYLOR, D. C. (1974a). Metabolism of chloroform. I. The metabolism of ^{14}C-chloroform by different species. *Xenobiotica* **4**, 151–163.

BROWN, B. R., JR., SIPES, I. G., AND SAGALYN, A. M. (1974b). Mechanisms of acute hepatic toxicity: Chloroform, halothane, and glutathione. ANESTHESIOLOGY **41**, 554–561.

BRUCKNER, J. V., MACKENZIE, W. F., MURALIDHARA, S., LUTHRA, R., KYLE, G. M., AND ACOSTA, D. (1986). Oral toxicity of carbon tetrachloride: Acute, subacute, and subchronic studies in rats. *Fundam. Appl. Toxicol.* **6**, 16–34.

BRUNIUS, G. (1987). Mitogenic activity of chloroform and carbon tetrachloride in serum-deficient or calcium-deficient cultures of human embryonic lung fibroblasts. *Carcinogenesis* **8**, 1645–1649.

BULL, R. J., BROWN, J. M., MEIERHENRY, E. A., JORGENSON, T. A., ROBINSON, M., AND STOBER, J. A. (1986). Enhancement of the hepatotoxicity of chloroform in B6C3F1 mice by corn oil: Implications for chloroform carcinogenesis. *Environ. Health Perspect.* **69**, 49–58.

BUREK, J. D., NITSCHKE, K. D., BELL, T. J., WACKERLE, D. L., CHILDS, R. C., BEYER, J. E., DITTENBER, D. A., RAMPY, L. W., AND MC KENNA, M. J. (1984). Methylene chloride: A 2-year inhalation toxicity and oncogenicity study in rats and hamsters. *Fundam. Appl. Toxicol.* **11**, 48–59.

BUTTERWORTH, B. E. (1989). Nongenotoxic carcinogens in the regulatory environment. *Regul. Toxicol. Pharmacol.* **9**, 244–256.

CHEESEMAN, K. H., ALBANO, E. F., TOMASI, A., AND SLATER, T. F. (1985). Biochemical studies on the metabolic activation of halogenated alkanes. *Environ Health Perspect.* **64**, 85–101.

CLAYSON, D. B., NERA, E. A., AND LOK, E. (1989). The potential for the use of cell proliferation studies in carcinogen risk assessment. *Regul. Toxicol. Pharmacol.* **9**, 284–295.

DANIELSEN, H., STEEN, H. B., LINDMO, T., AND REITH, A. (1988). Ploidy distribution in experimental liver carcinogenesis in mice. *Carcinogenesis* **9**, 59–63.

DEAL, F. H., RICHARDSON, F. C., AND SWENBERG, J. A. (1989). Dose response of hepatocyte replication in rats following continuous exposure to diethylnitrosamine. *Cancer Res.* **49**, 6985–6988.

DEWANJI, A., VENZON, D. J., AND MOOLGAVKAR, S. H. (1989). A stochastic two-stage model for cancer risk assessment. II. The number and size of premalignant clones. *Risk Analysis* **9**, 179–187.

DINMAN, B. D., HAMDI, E. A., FOX, C. F., AND FRAJOLA, W. J. (1963). CCl_4 toxicity. *Arch. Environ. Health* **7**, 630–646.

DOBSON, R. L., AND FELTON, J. S. (1983). Female germ cell loss from radiation and chemical exposures. *Amer. J. Ind. Med.* **4**, 175–190.

DOOLITTLE, D. J., MULLER, G., AND SCRIBNER, H. E. (1987). Relationship between hepatotoxicity and induction of replicative DNA synthesis following single or multiple doses of carbon tetrachloride. *J. Toxicol. Environ. Health* **22**, 63–78.

DRAGANI, T. A., MANENTI, G., AND PORTA, G. D. (1986). Enhancing effects of carbon tetrachloride in mouse hepatocarcinogenesis. *Cancer Lett.* **31**, 171–179.

FEINGOLD, A., AND HOLIDAY, D. A. (1977). The pharmacokinetics of metabolism of inhalation anaesthetics. *Brit. J. Anaesth.* **49**, 155–162.

FINNEY, D. J. (1952). *Statistical Methods in Biological Assay*. Hafner, New York.

FRY, J., TAYLOR, T., AND HATHAWAY, D. F. (1972). Pulmonary elimination of chloroform and its metabolite in man. *Arch. Int. Pharmacodyn.* **196**, 98–111.

GARGAS, M. L., ANDERSEN, M. E., AND CLEWELL, H. J., III (1986a). A physiologically based simulation approach for determining metabolic constants from gas uptake data. *Toxicol. Appl. Pharmacol.* **86**, 341–352.

GARGAS, M. L., ANDERSEN, M. E., AND CLEWELL, H. J., III (1986b). Metabolism of inhaled dihalomethanes *in vivo*: Differentiation of kinetic constants for two independent pathways. *Toxicol. Appl. Pharmacol.* **87**, 211–223.

GARGAS, M. L., SEYBOLD, P. G., AND ANDERSEN, M. E. (1988). Modelling the tissue solubilities and metabolic rate constant (V_{max}) of halogenated methanes, ethanes, and ethylenes. *Toxicol. Lett.* **43**, 235–256.

GARGAS, M. L., BURGESS, R. J., VOISSARD, D. E., CASON, G. H., AND ANDERSEN, M. E. (1989). Partition coefficients of low molecular weight volatile chemicals in various liquids and tissues. *Toxicol. Appl. Pharmacol.* **98**, 87–99.

GERHARD, H. (1973). A quantitative model of cellular regeneration in rat liver after partial hepatectomy. In *Liver Regeneration After Experimental Injury: III. Workshop on Experimental Liver Injury* (R. Lesch and W. Reutter, Eds.), pp. 340–346. Stratton Intercontinental Medical Book Corp., New York.

GOCKE, E., KING, M. T., ECKHARDT, K., AND WILD, D. (1981). Mutagenicity of cosmetics ingredients licensed by the European communities. *Matat. Res.* **90**, 91–109.

GREEN, T., PROVAN, W. M., COLLINGE, D. C., AND GUEST, A. E. (1988). Macromolecular interactions of inhaled methylene chloride in rats and mice. *Toxicol. Appl. Pharmacol.* **93**, 1–10.

GRISHAM, J. W., AND SMITH, G. J. (1984). Predictive and mechanistic evaluation of toxic responses in mammalian cell culture systems. *Pharmacol. Rev.* **36**, 151S–171S.

HOY, C. A., THOMPSON, L. H., MOONEY, C. L., AND SALAZAR, E. P. (1985). Defective DNA cross-link removal in Chinese hamster cell mutants hypersensistive to bifunctional alkylating agents. *Cancer Res.* **45**, 1737–1743.

JLETT, K. F., REID, W. D., SIPES, I. G., AND KRISHNA, G. (1973). Chloroform toxicity in mice: Correlation of renal and hepatic necrosis with covalent binding of

metabolites to tissue macromolecules. *Exp. Mol. Pathol.* **19**, 215–229.

International Agency for Research on Cancer (IARC) (1979a). *IARC Monogr. Eval. Carcinog. Risk Chem. Man* **20**, 371–399.

International Agency for Research on Cancer (IARC) (1979b). *IARC Monogr. Eval. Carcinog. Risk Chem. Man* **20**, 401–427.

International Agency for Research on Cancer (IARC) (1982). *IARC Monogr. Eval. Carcinog. Risk Chem. Man Suppl.* **4** (Lyon, France), 87–88.

International Agency for Research on Cancer (IARC) (1986). *IARC Monogr. Eval. Carcinog. Risk Chem. Man* **41**, 43–85.

JONGEN, W. M. F., LOHMAN, P. M. H., KOTTENHAGE, M. J., ALINK, G. M., BERENDS, F., AND KOEMAN, J. H. (1981). Mutagenicity testing of dichloromethane in short-term mammalian test systems. *Mutat. Res.* **81**, 203–213.

KIRSCHMAN, J. C., BROWN, N. M., AND COOTS, R. H. (1986). Review of Investigations of dichloromethane metabolism and subchronic oral toxicity as the basis for the design of chronic oral studies in rats and mice. *Food Chem. Toxicol.* **24**, 943–949.

KITCHIN, K. T., AND BROWN, J. L. (1989). Biochemical effects of three carcinogenic chlorinated methanes in rat livers. *Teratog. Carcinog. Mutagen.* **9**, 61–69.

KLASSEN, C. D., AND PLAA, G. L. (1966). Relative effects of various chlorinated hydrocarbons on liver and kidney function in mice. *Toxicol. Appl. Pharmacol.* **9**, 139–151.

KUBIC, V. L., ANDERS, M. W., ENGEL, P. R., BARLOW, C. H., AND CAUGHEY, W. S. (1974). Metabolism of dihalomethanes to carbon monoxide. *Drug Metab. Dispos.* **2**, 53–57.

LABBE, G., DESCATOIRE, V., LETTERON, P., DEGOTT, C., TINEL, M., LARREY, D., CARRION-PAVLOV, Y., AMOUYAL, J. G. G., AND PESSAYRE, D. (1987). The drug methoxsalen a suicide substrate for cytochrome P-450. *Biochem. Pharmacol.* **36**, 907–914.

LANDOLPH, J. R., BARTHOLOMEW, J. C., AND CALVIN, M. (1976). Quantitative studies of the toxicity of benzo[*a*]pyrene to a mouse liver epithelial cell strain in culture. *Cancer Res.* **36**, 4143–4151.

LAVIGNE, J. G., AND MARCHAND, C. (1974). The role of metabolism in chloroform hepatotoxicity. *Toxicol. Appl. Pharmacol.* **29**, 312–326.

LEEVY, C. M., HOLLISTER, R. M., SCHMID, R., MACDONALD, R. A., AND DAVIDSON, C. S. (1959). Liver regeneration in experimental carbon tetrachloride intoxication. *Proc. Soc. Exp. Biol. Med.* **102**, 672–675.

LEFEVRE, P. A., AND ASHBY, J. (1989). Evaluation of dichloromethane as an inducer of DNA synthesis in the $B_6C_3F_1$ mouse liver. *Carcinogenesis* **10**, 1067–1072.

LEVINE, R. R. (1983). *Pharmacology: Drug Actions and Reactions*, Little, Brown, Boston. pp. 175–209.

LICKO, V. (1972). Some biochemical threshold mechanisms. *Bull. Math. Biophys.* **34**, 103–112.

LONG, R. M., AND MOORE, L. (1986). Elevated cytosolic calcium in rat hepatocytes exposed to carbon tetrachloride. *J. Pharmacol. Exp. Ther.* **238**, 186–191.

MCMARTIN, D. N., O'CONNER, J. A., AND KAMINSKY, L. S. (1981). Effects of differential changes in rat hepatic and renal cytochrome P-450 concentrations on hepatotoxicity and nephrotoxicity to chloroform. *Res. Commun. Chem. Pathol. Pharmacol.* **31**, 99–111.

MINK, F. L., BROWN, T. J., AND RICKABAUGH, J. (1986). Absorption, distribution, and excretion of ^{14}C-trihalomethanes in mice and rats. *Bull. Environ. Contam. Toxicol.* **37**, 752–758.

MIRSALIS, J. C., AND BUTTERWORTH, B. E. (1980). Detection of unscheduled DNA synthesis in hepatocytes isolated from rats treated with genotoxic agents: an *in vivo–in vitro* assay for potential carcinogens and mutagens. *Carcinogenesis* **1**, 621–625.

MIRSALIS, J. C., TYSON, C. K., AND BUTTERWORTH, B. E. (1982). Detection of genotoxic carcinogens in the *in vivo–in vitro* DNA repair assay. *Environ. Mutagen.* **4**, 553–562.

MIRSALIS, J. C., TYSON, C. K., LOH, E. N., STEINMETZ, K. L., BAKKE, J. P., HAMILTON, C. M., SPAK, D. K., AND SPALDING, J. W. (1985). Induction of hepatic cell proliferation and unscheduled DNA synthesis in mouse hepatocytes following *in vivo* treatment. *Carcinogenesis* **6**, 1521–1524.

MOOLGAVKAR, S. H., AND VENZON, D. J. (1979). Two-event models for carcinogenesis: Incidence curves for childhood and adult tumors. *Math. Biosci.* **47**, 55–77.

MOOLGAVKAR, S. H., AND KNUDSON, A. G. (1981). Mutation and cancer: A model for human carcinogenesis. *J. Natl. Cancer Inst.* **66**, 1037–1052.

MOOLGAVKAR, S. H. (1983). Model for human carcinogenesis: Action of environmental agents. *Environ. Health Perspect.* **50**, 285–291.

MOOLGAVKAR, S. H., DAY, N. E., AND STEVENS, R. G. (1980). Two-stage model for carcinogenesis: Epidemiology of breast cancer in females. *J. Natl. Cancer Inst.* **65**, 559–569.

MOOLGAVKAR, S. H., DEWANJI, A., AND VENZON, D. J. (1988). A stochastic two-stage model for cancer risk assessment. I. The hazard function and the probability of tumor. *Risk Analysis* **8**, 383–392.

MOORE, L. (1980). Inhibition of liver-microsome calcium pump by *in vivo* administration of CCl_4, $CHCl_3$, and 1,1-dichloroethylene (vinylidene chloride). *Biochem. Pharmacol.* **29**, 2505–2511.

MOORE, D. H., CHASEAUD, L. F., MAJEED, S. K., PRENTICE, D. E., AND ROE, J. C. (1982). The effect of dose

and vehicle on early tissue damage and regeneration activity after chloroform administration to mice. *Food. Chem. Toxicol.* **20**, 951–954.

National Academy of Sciences, National Research Council. Committee on the Biological Effects of Ionizing Radiations (NAS) (1980). *The Effects on Populations of Exposure to Low Levels of Ionizing Radiation.* National Academy of Sciences, Washington, D.C.

National Cancer Institute (NCI) (1976a). *Report on the Carcinogenesis Bioassay of Chloroform.* Carcinogenesis Program, Division of Cancer Cause and Prevention, Bethesda, MD.

National Cancer Institute (NCI) (1976b). *Report on the Carcinogenesis Bioassay of Trichloroethylene.* National Cancer Institute Technical Report Series, No. 2. NCI-CG-TR-2. Bethesda, MD.

National Cancer Institute (NCI) (1977). *Bioassay of 1,1,1-Trichloroethane for Possible Carcinogenicity.* National Cancer Institute Technical Report Series, No. 3. NCI-CG-TR-3. Bethesda, MD.

National Council on Radiological Protection and Measurements (NCRP) (1989). *Comparative Carcinogenicity of Ionizing Radiation and Chemicals.* NCRP Report No. 96. Bethesda, MD.

National Toxicology Program (NTP) (1986). *Toxicology and Carcinogenesis Studies of Dichloromethane (Methylene Chloride) in F344/N Rats and B6C3F1 Mice (Inhalation Studies).* NIH Publ. No. 86-2562, NTP TR 306. U.S. Department of Health and Human Services, National Institutes of Health, Research Triangle Park, NC.

Nitschke, K. D., Burek, J. D., Bell, T. J., Kociba, R. J., Rampy, L. W., and McKenna, M. J. (1988). Methylene chloride: A 2-year inhalation toxicity and oncogenicity study in rats. *Fundam. Appl. Toxicol.* **11**, 48–59.

Ottenwalder, H., and Peter, H. (1989). DNA binding assay of methylene chloride in rats and mice. *Arch. Toxicol.* **63**, 162–163.

Paustenbach, D. J., Clewell, H. J., III, Gargas, M. L., and Andersen, M. E. (1988). A physiologically based pharmacokinetic model for inhaled carbon tetrachloride. *Toxicol. Appl. Pharmacol.* **96**, 191–211.

Pereira, M. A., Savage, R. E., Jr., Guion, C. W., and Wernsing, P. A. (1984). Effect of chloroform on hepatic and renal DNA synthesis and ornithine decarboxylase activity in mice and rats. *Toxicol. Lett.*, **21**, 357–364.

Perocco, P., and Prodi, G. (1981). DNA damage by halokanes in human lymphocytes cultured *in vitro*. *Cancer Lett.* **13**, 213–218.

Petersen, S. (1985). Flow cytometry of human colorectal tumors: Nuclear isolation by detergent technique. *Cytometry* **6**, 452–460.

Peterson, A. R., and Peterson, H. (1985). Estimation of the potencies of chemicals that produce genetic damage. *Carcinogenesis* **10**, 495–508.

Portier, C. J. (1987). Statistical properties of a two-stage model of carcinogenesis. *Environ. Health Perspect.* **76**, 125–131.

Rabes, H. M., Tuczek, H. V., and Wirsching, R. (1973). Kinetics of hepatocellular proliferation after partial hepatectomy as a function of structural and biochemical heterogeneity of the rat liver. In *Liver Regeneration after Experimental Injury: III. Workshop on Experimental Liver Injury* (R. Lesch and W. Reutter, Eds.), pp. 35–55. Stratton Intercontinental Medical Book Corp., New York.

Raje, R., Basso, M., Tolen, T., and Greening, M. (1988). Evaluation of *in vivo* mutagenicity of low-dose methylene chloride in mice. *J. Amer. Coll. Toxicol.* **7**, 699–703.

Ramsey, J. C., and M. E. Andersen (1984). A physiologically based description of the inhalation pharmacokinetics of styrene in rats and humans. *Toxicol. Appl. Pharmacol.* **73**, 159–175.

Ray, P., and Moore, L. (1986). Carbon tetrachloride-induced release of calcium from isolated hepatocytes. *Toxicology* **41**, 205–212.

Reitz, R. H., Fox, T. R., and Quast, J. F. (1982). Mechanistic considerations for carcinogenic risk estimation: chloroform. *Environ. Health Perspect.* **46**, 163–168.

Reitz, R. H., Smith, F. A., and Andersen, M. E. (1986). *In vivo* metabolism of ^{14}C-methylene chloride (MEC). *Toxicologist* **6**, 260.

Reitz, R. H., Mendrala, A. L., Park, C. N., Andersen, M. E., and Guengerich, F. P. (1988). Incorporation of *in vitro* enzyme data into the physiologically based pharmacokinetic (PB–PK) model of methylene chloride: Implications for risk assessment. *Toxicol. Lett.* **43**, 97–116.

Reitz, R. H., Mendrala, A. L., and Guengerich, F. P. (1989). *In vitro* metabolism of methylene chloride in human and animal tissues: Use in physiologically based pharmacokinetic models. *Toxicol. Appl. Pharmacol.* **97**, 230–246.

Reuber, M. D. (1979). Carcinogenicity of chloroform. *Environ. Health Perspect.* **31**, 171–182.

Reynolds, E. S. (1967). Liver parenchymal cell injury. IV. Pattern of incorporation of carbon and chlorine from carbon tetrachloride into chemical constituents of liver *in vivo*. *J. Pharmacol. Exp. Ther.* **115**, 117–126.

Reynolds, E. S., Treinen, R. J., Farrish, H. H., and Moslen, M. T. (1984). Metabolism of [^{14}C] carbon tetrachloride to exhaled, excreted and bound metabolites: Dose–response, time-course and pharmacokinetics. *Biochem. Pharmacol.* **33**, 3363–3374.

ROGHANI, M., DA SILVA, C., AND CASTAGNA, M. (1987). Tumor promoter chloroform is a potent protein kinase C activator. *Biochem. Biophys. Res. Commun.* **142**, 738–744.

ROSENTHAL, S. L. (1987). A review of the mutagenicity of chloroform. *Environ. Mol. Mutagen.* **10**, 211–226.

SATO, A., AND NAKAJIMA, T. (1979). A structure–activity relationship of some chlorinated hydrocarbons. *Arch. Environ. Health* **34**, 69–75.

SCHULTZE, B., GERHARD, H., AND MAURER, W. (1973). A quantitative model of liver regeneration in the mouse after CCl_4 intoxication. In Lesch, R., and Reutter, W., Eds. *Liver Regeneration after Experimental Injury: III. Workshop on Experimental Liver Injury* (R. Lesch and W. Reutter, Eds.), pp. 330–339. Stratton Intercontinental Medical Book Corp., New York.

SHELDON, T., RICHARDSON, C. R., AND ELLIOT, B. M. (1987). Inactivity of methylene chloride in the mouse bone marrow micronucleus assay. *Mutagenesis* **2**, 57–59.

SHERTZER, H. G., NIEMI, M. P., REITMAN, F. A., BERGER, M. L., MYERS, B. L., AND TABOR, M. W. (1987). Protection against carbon tetrachloride hepatotoxicity by pretreatment with indole-3-carbinol. *Exp. Mol. Pathol.* **46**, 180–189.

SHERTZER, H. G., BERGER, M. L., AND TABOR, M. W. (1988). Intervention in free radical mediated hepatotoxicity and lipid. *Biochem. Pharmacol.* 37, 333–338.

SMUCKLER, E. A., AND JAMES, J. L. (1984). Irreversible cell injury. Pharmacol. Rev. **36**, 77S–91S.

STEWARD, A., ALLOT, P. R., COWLES, A. L., AND MAPLESON, W. W. (1973). Solubility coefficients for inhaled anesthetics for water, oil, and biological media. *Brit. J. Anaesth.* **45**, 282–293.

SWENBERG, J. A., RICHARDSON, F. C., BOUCHERON, J. A., DEAL, F. H., BELINSKY, S. A., CHARBONNEAU, M., AND SHORT, B. G. (1987). High- to low-dose extrapolation: Critical determinants involved in the dose response of carcinogenic substances. *Environ. Health Perspect.* **76**, 57–63.

TANAKA, T., MORI, H., AND WILLIAMS, G. M. (1987). Enhancement of dimethylnitrosamine-initiated hepatocarcinogenesis in hamsters by subsequent administration of carbon tetrachloride but not phenobarbital or *p,p'*-dichlorodiphenyltrichloroethane. *Carcinogenesis* **8**, 1171–1178.

TAYLOR, D. C., BROWN, D. M., KUBLE, R., AND LANGLEY, P. F. (1974). Metabolism of chloroform. II. A sex difference in the metabolism of ^{14}C-chloroform in mice. *Xenobiotica* **4**, 165–174.

THILAGAR, A. K., AND KUMAROO, P. V. (1983). Induction of chromosome damage by methylene chloride in CHO cells. *Mutat. Res.* **116**, 361–367.

THILAGAR, A. K., KUMAROO, P. V., CLARKE, J. J., KOTT, S., BACK, A. M., AND KIRBY, P. E. (1984). Induction of chromosome damage by dichloromethane in cultured human peripheral lymphocytes, CHO cells and mouse lymphoma L5178Y cells. *Environ. Mutagen.* **6**, 442 [Abstract].

THOMPSON, L. H., SALAZAR, E. L., BROOKMAN, K. W., AND HOY, C. A. (1983). Hypersensitivity to cell killing and mutation induction by chemical carcinogens in an excision-repair deficient mutant of CHO cells. *Mutat. Res.* **112**, 329–344.

THORSLAND, T. W., BROWN, C. C., AND CHARNLEY, G. (1987). Biologically motivated cancer risk models. *Risk Analysis* **7**, 109–119.

TOMASI, A., ALBANO, E., BANNI, S., BOTTI, B., CORONGIU, F., DESSI, M. A., IANNONE, A., VANNINI, V., AND DIANZANI, M.U. (1987). Free-radical metabolism of carbon tetrachloride in rat liver mitochondria. *Biochem. J.* **246**, 313–317.

TRUEMAN, R. W., AND ASHBY, J. (1987). Lack of UDS activity in the livers of mice and rats exposed to dichloromethane. *Environ. Mutagen.* **10**, 189–195.

U.S. Environmental Protection Agency (EPA) (1980). Water quality criteria documents. *Fed. Regist.* **45**, 79,350–79,351.

U.S. Environmental Protection Agency (EPA) (1984). *Health Assessment Document for Carbon Tetrachloride.* EPA-600/8-82-001F. U.S. EPA Environmental Criteria and Assessment Office, Cincinnati OH.

U.S. Environmental Protection Agency (EPA) (1985a). *Health Assessment Document for Chloroform.* EPA/600/8-84/004F. U.S. EPA Office of Health and Environmental Assessment, Washington, DC.

U.S. Environmental Protection Agency (EPA) (1985b). *Health Assessment Document for Dichloromethane (Methylene Chloride).* EPA/600/8-82/004F. U.S. EPA Office of Health and Environmental Assessment, Washington, DC.

U.S. Environmental Protection Agency (EPA) (1985c). *Addendum to the Health Assessment Document for Dichloromethane (Methylene Chloride): Updated. Carcinogenicity Assessment of Dichloromethane (Methylene Chloride).* EPA/600/8-82/004F. U.S. EPA Office of Health and Environmental Assessment, Washington, DC.

U.S. Environmental Protection Agency (EPA) (1986). Guidelines for carcinogen risk assessment. *Fed. Regist.* **51**, 33,992–34,003.

U.S. Environmental Protection Agency (EPA) (1987). *Update to the Health Assessment Document and Addendum for Dichloromethane (Methylene Chloride): Pharmacokinetics, Mechanism of Action, and Epidemiology.* EPA/600/8-82/004F. U.S. EPA Office of Health and Environmental Assessment, Washington, DC.

U.S. Environmental Protection Agency (EPA) (1988). *Reference Physiological Parameters in Pharmacoki-*

netic Modeling. EPA-600/6-88/004, NTIS PB88-196019. U.S. EPA Office Research and Development, Office of Health and Environmental Assessment, Washington, DC.

VILLARRUEL, M. C., FERNANDEZ, G., AGUILAR, E. G., AND CASTRO, J. A. (1987). Early biochemical alterations in liver mitochondria from carbon tetrachloride poisoned rats. *J. Appl. Toxicol.* **7**, 173–177.

WEINSTEIN, R. S., BOYD, D. D., AND BACK, K. C. (1972). Effects of continuous inhalation of dichloromethane in the mouse: Morphologic and functional observations. *Toxicol. Appl. Pharmacol.* **23**, 660–679.

WEISBURGER, J. H., AND WILLIAMS, G. M. (1983). The distinct health risk analyses required for genotoxic carcinogens and promoting agents. *Environ. Health Perspect.* **50**, 233–245.

WILLIAMS, G. M. (1967). Definition of a human cancer hazard. In *Nongenotoxic Mechanisms in Carcinogenesis* (Banbury Report 25) (B. E. Butterworth and T. J. Slaga, Eds.), pp. 367–380. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

WILSON, J. D. (1988). Risk assessment reappraisals. *Science* **240**, 1126 [Letter].

WITHEY, J. R., COLLINS, B. T., COLLINS, P. G. (1983). Effect of vehicle on the pharmacokinetics and uptake of four halogenated hydrocarbons from the gastrointestinal tract of the rat. *J. Appl. Toxicol.* **3**, 249–253.